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Hemoglobin (Hb) has been demonstrated to be neurotoxic when injected into the cerebral cortex, in vivo. However, associated systemic factors such as ischemia and epileptogenesis have limited investigations of Hb toxicity in the intact central nervous system (CNS). In this study, the neurotoxicity of human Hb was assessed in mixed neuronal and glial neocortical cell cultures derived from fetal mice. Exposure of cultures to Hb for 24-28 hours produced widespread and concentration-dependent neuronal death (EC_{50} 1-2.5 μM), without injuring glia. Brief exposures (1-2 hours) were not toxic. Neuronal death was completely blocked by the 21-aminosteroid U74500A, the antioxidant Trolox, and the ferric iron chelator deferoxamine. The results of these experiments suggest that, in this system, chromatographically pure Hb is a potent neurotoxin, and that Hb neurotoxicity may contribute to secondary injury processes after trauma and intracranial hemorrhage.

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NEUROTOXICITY OF HEMOGLOBIN IN CORTICAL CELL CULTURE

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Key Words: hemoglobin, neuronal culture, iron, antioxidants, chelators, toxicity, deferoxamine, Trolox

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SUMMARY:

Hemoglobin (Hb) has been demonstrated to be neurotoxic when injected into the cerebral cortex, in vivo. However, associated systemic factors such as ischemia and epileptogenesis have limited investigations of Hb toxicity in the intact central nervous system (CNS). In this study, the neurotoxicity of human Hb was assessed in mixed neuronal and glial neocortical cell cultures derived from fetal mice. Exposure of cultures to Hb for 24-28 hours produced widespread and concentration-dependent neuronal death (EC_{50} 1-2.5 μM), without injuring glia. Brief exposures (1-2 hours) were not toxic. Neuronal death was completely blocked by the 21-aminosteroid U74500A, the antioxidant Trolox, and the ferric iron chelator deferoxamine. The results of these experiments suggest that, in this system, chromatographically pure Hb is a potent neurotoxin, and that Hb neurotoxicity may contribute to secondary injury processes after trauma and intracranial hemorrhage.

Exposure of central neurons to extravascular blood occurs in many acute injury processes, including intraparenchymal hemorrhages, contusions, and hemorrhagic infarctions. Much of the neuronal death occurring in these processes has been attributed to the primary injury or to secondary ischemia, but a direct neurotoxic effect of blood has also been hypothesized [19, 20]. A possible mediator of neurotoxicity is hemoglobin (Hb), which is highly concentrated in erythrocytes and accounts for most of the 8-10 millimolar iron concentration in whole blood.

Under normal circumstances, free Hb is effectively sequestered by haptoglobin and does not reach toxic levels [8, 9] . However, in some situations, the release of large amounts of Hb from lysed erythrocytes may saturate haptoglobin, exposing neurons and glia to Hb and its breakdown products. In vivo studies support a possible neurotoxic effect of parenchymal Hb; in rats, intracortical injection produces cavitary lesions and gliosis at injection sites [19]. However, Hb neurotoxicity is difficult to quantify in vivo due to the potent vasospasm [16] and seizure activity [19] that may accompany its administration. In the present study, we utilized a primary murine neocortical cell culture system to investigate Hb neurotoxicity in an environment free of complicating systemic variables.

Human Hb, purified by high pressure liquid chromatography according to the technique of Christensen et al. [5], was prepared in the Hb production facility at the Letterman Army Institute of Research [28]. The final product was sterile filtered, formulated in Ringer's acetate, and stored at -80° C. Hemoglobin concentrations were determined using Drabkin's solution [26] and metHb concentrations at the time of use were determined to be less than 4.0% according to the method of Evelyn and Malloy [6, 11]. Endotoxin concentration was determined to be < 0.1 EU/ml. All Hb concentrations are expressed as the concentration of tetramer.

Mixed cortical cell cultures, containing both neurons and glia, were prepared with modification of methods described by Choi et al. [4]. After halothane anesthesia, pregnant Swiss-Webster mice (15-16 days gestation) were euthanized by cervical dislocation. Embryos were rapidly removed and decapitated, and the neocortex was dissected free and incubated in medium containing 0.09% acetylated trypsin at 37°C for one hour. Tissue was then suspended in plating medium consisting of Eagle's minimal essential medium (MEM), 5% heat-inactivated horse serum, 5% fetal bovine serum, glutamine (2mM), and glucose (23 mM). After trituration through a flame-polished Pasteur pipette, cells were diluted in additional plating medium and seeded on confluent glial cultures in 24-well multiwell plates (Falcon) at a density of 2.2 hemispheres/plate. Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Culture medium was partially changed twice weekly with medium containing 10% horse serum and lacking fetal bovine serum. Nonneuronal cell division was inhibited at 6-9 days in vitro (DIV) by addition of 10^{-5} M cytosine arabinoside.

Cortical glial cultures were prepared from Swiss-Webster mice at postnatal day 1-3. After halothane anesthesia, mice were decapitated, and heads were submerged in 70% ethanol for 3-4 minutes. Dissection and dissociation were as described above for mixed cultures, and cells were diluted in plating medium containing10% fetal bovine serum, 10% horse serum, 2 mM glutamine, and epidermal growth factor (10 ng/ml). The cell suspension was plated on 24-well Primaria (Falcon) multiwell plates at a density of 0.5 hemisphere per plate.

Exposure of mixed neuronal and glial cultures to Hb was carried out at 13-16 DIV in a defined solution consisting of MEM plus glucose (25 mM), which was substituted for culture medium by triple exchange. After addition of Hb alone or with drugs, cultures were

incubated at 37°C in a 5% CO_2 atmosphere. Neuronal injury was estimated in all experiments by examination of cultures with phase-contrast microscopy at 100-200x and was quantified by measurement of lactate dehydrogenase (LDH) in the culture medium at the end of the exposure period. [13].

Exposure of cultures to Hb for 24-28 hours produced significant neuronal injury that was concentration dependent between 0.025 and 25 μM (EC_{50} 1-2.5 μM). Exposure to 25 μM Hb resulted in degeneration of 80-100 % of neurons (Fig. 1). The underlying glial monolayer, in contrast, remained intact and appeared morphologically normal through several days of exposure. Experiments using pure glial cultures revealed no evidence of Hb-induced glial injury or LDH release.

Brief exposure to Hb was consistently well tolerated by both neurons and glia. A one hour incubation in 25 μM Hb followed by washout into MEM produced little neuronal injury over the subsequent 23 hours (Fig. 2). Some variation was noted in the exposure time required to produce neuronal injury, particularly between cultures prepared with different lots of serum. In most experiments, many neurons were swollen after an eight hour exposure period, and subsequently degenerated over the next several hours.

To test the hypothesis that Hb neurotoxicity is mediated by oxidation of cellular components, we exposed cultures to Hb in the presence of Trolox or the 21-aminosteroid U74500A (Fig. 2). Trolox, a water-soluble analog of α-tocopherol [29], consistently attenuated the neuronal injury produced by 24-28 hour exposure to 25 μM Hb, with nearly complete protection noted at 10 μM Trolox (Fig. 3). U74500A, an inhibitor of lipid peroxidation which may also chelate iron [3], was likewise effective but more potent. Significant neuroprotection was noted at 100 nM and was complete at 1 μM. At 10 nM, no

effect was seen. U74500A was prepared in a stock solution containing dimethylsulfoxide (DMSO). No significant effect was noted from the DMSO vehicle alone at the concentration used in these experiments (0.1%).

Lipid peroxidation can be initiated by highly reactive hydroxyl radical (OH·), and one mechanism of OH· formation is via the iron-catalyzed Haber-Weiss reaction [2]. To assess the role of OH· formation in Hb neurotoxicity, we used deferoxamine, which is a ferric iron chelator that also scavenges OH· [10]. Neuronal injury was blocked by deferoxamine in a concentration-dependent fashion, and near complete protection was provided by 10 μM (Fig. 4).

The precise molecular events precipitating neuronal death after Hb exposure remain undefined. Hemoglobin is capable of releasing reactive iron that can catalyze the formation of OH· [21, 7]. Once formed, OH· would most likely react in the immediate vicinity of the site of production and, if formed in the extracellular space, would have limited access to the intracellular organelles [24]. Interaction of OH· with cell membrane phospholipids may initiate free radical chain reactions within the membrane, leading to decreased membrane fluidity and loss of membrane integrity [23]. Alternatively, OH· may interfere with the activity of membrane-bound enzymes, either by direct protein oxidation or disruption of surrounding membrane phospholipids. Anderson and Means reported inhibition of spinal cord Na^+/K^+ ATPase in vivo by iron salts, and this effect was prevented by high dose methylprednisolone, α-tocopherol, or selenium [1]. Leclerc et al., noted potent inhibition of erythrocyte membrane Ca^{2+}/Mg^{2+}ATPase in vitro by 100 μM ferric heme or nonheme iron [14, 15]. Even partial inhibition of membrane cation pumps may have dire consequences for neurons, which must maintain steep ionic gradients and recover promptly from depolarization.

Regardless of the exact mechanism of Hb-dependent neuronal injury, the results of the current experiments suggest that exposure of neurons to Hb may be undesirable and that it may be beneficial to clear extravascular Hb as rapidly as possible. Hemoglobin is normally cleared by the plasma protein haptoglobin, which forms an irreversible complex with Hb and facilitates its uptake by the liver [12]. This clearance process is extremely efficient; the plasma half-life of haptoglobin alone in human plasma is 3.5 days [12]. However, when bound to Hb, the plasma half-life of the complex is approximately 10 minutes [12]. In addition to assisting the clearance of free Hb, haptoglobin also attenuates the pro-oxidant effects of Hb [8]. Thus, haptoglobin may play an important role in the control of Hb-dependent tissue damage.

In the present experiments, exposure of cultures to a Hb concentration approximating 1% of that present in human blood produced marked neuronal injury. These results should be extended to situations in vivo with some caution. During the course of these experiments, neurons were deprived of the usual antioxidant defenses normally present in extracellular fluid, e.g., plasma or cerebrospinal fluid [25, 27]. The presence of these constituents may counterbalance the loss of intracellular central nervous system (CNS) antioxidants such as reduced glutathione, α-tocopherol, and ascorbate, all of which decline precipitously after trauma and ischemia [17, 18, 22]. However, high Hb concentrations may eventually overwhelm any remaining endogenous defenses and produce neurotoxicity. Pharmacologic strategies aimed at attenuating such injury may be of value in the management of CNS trauma and hemorrhage.

The opinions and assertions contained herein are the private views of the authors and are not to be construed as official, nor do they reflect the views of the Department of the Army or the Department of Defense (AR 360-5).

The experimental studies of the authors described in this report were reviewed and approved by the Institutional Review Committee/Animal Care and Use Committee at Letterman Army Institute of Research. The manuscript was peer reviewed for compliance prior to submission for publication. In conducting the research described here, the author adhered to the "Guide for the Care and Use of Laboratory Animals," DHEW Publication (NIH) 85-23.

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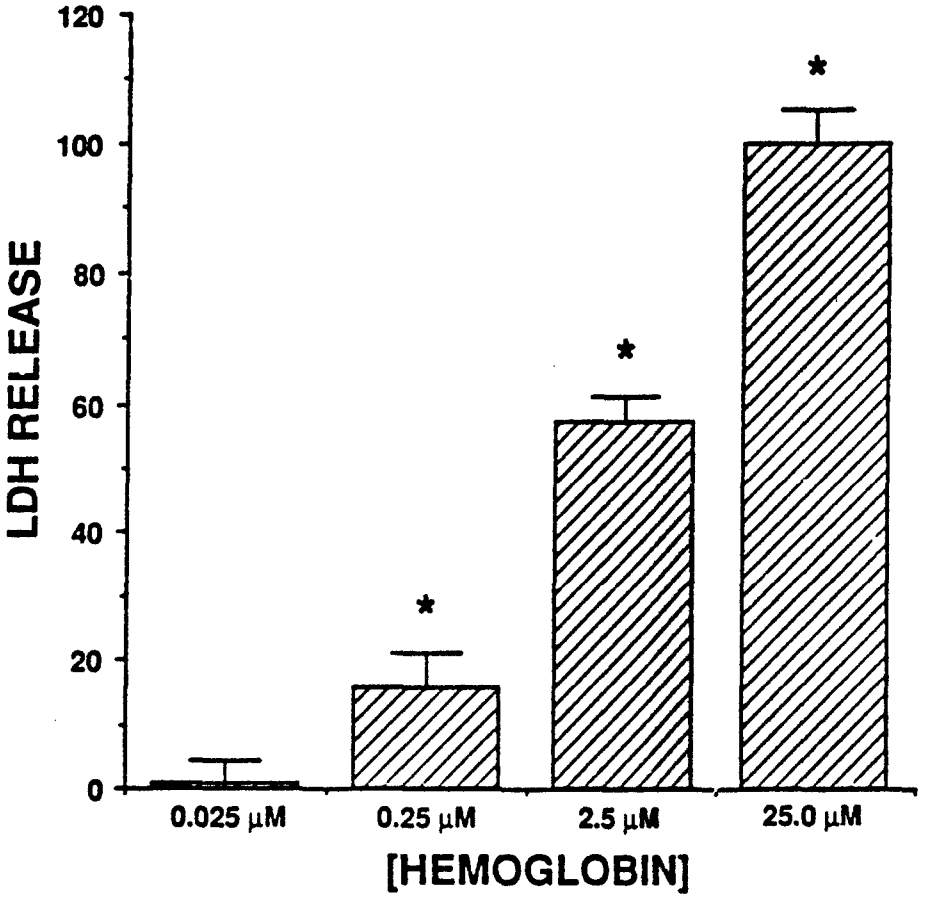
FIGURE LEGENDS

Fig. 1. Concentration dependence of hemoglobin neurotoxicity. Sister cultures were exposed to indicated concentrations of hemoglobin for 24-28 hours. LDH in the culture media was measured at the end of the experiment (mean + S.E.M., $n = 8$ cultures for each condition). LDH in this and subsequent figures was scaled to the mean value released by cultures exposed to 25 μM hemoglobin (100%). Asterisk indicates significant difference from sham wash control ($P < 0.05$, Student-Newman-Keuls test).

Fig. 2. Time course of hemoglobin neurotoxicity. Sister cultures were exposed to hemoglobin 25 μM for indicated time. LDH in the culture media was measured prior to washout and at 24 hours, and results were summed (mean + S.E.M., $n = 4$ for each condition).

Fig. 3. Antioxidant blockade of hemoglobin neurotoxicity. Sister cultures were exposed to hemoglobin 25 μM for 24-28 hours either alone (CTRL) or in the presence of indicated concentrations of Troloxor U74500A. LDH in the culture media was measured at the end of the exposure period (mean + S.E.M., $n = 8$ for each condition). Asterisk indicates significant difference from control ($P < 0.05$, Student-Newman-Keuls test).

Fig. 4. Iron dependence of hemoglobin neurotoxicity. Media LDH (mean ± S.E.M., $n = 8$) in sister cultures after 24-28 hour exposure to hemoglobin 25 μM in the presence of indicated concentrations of deferoxamine, scaled to LDH released in control cultures exposed to hemoglobin alone (=100). Approximate IC_{50} for deferoxamine = 3μM.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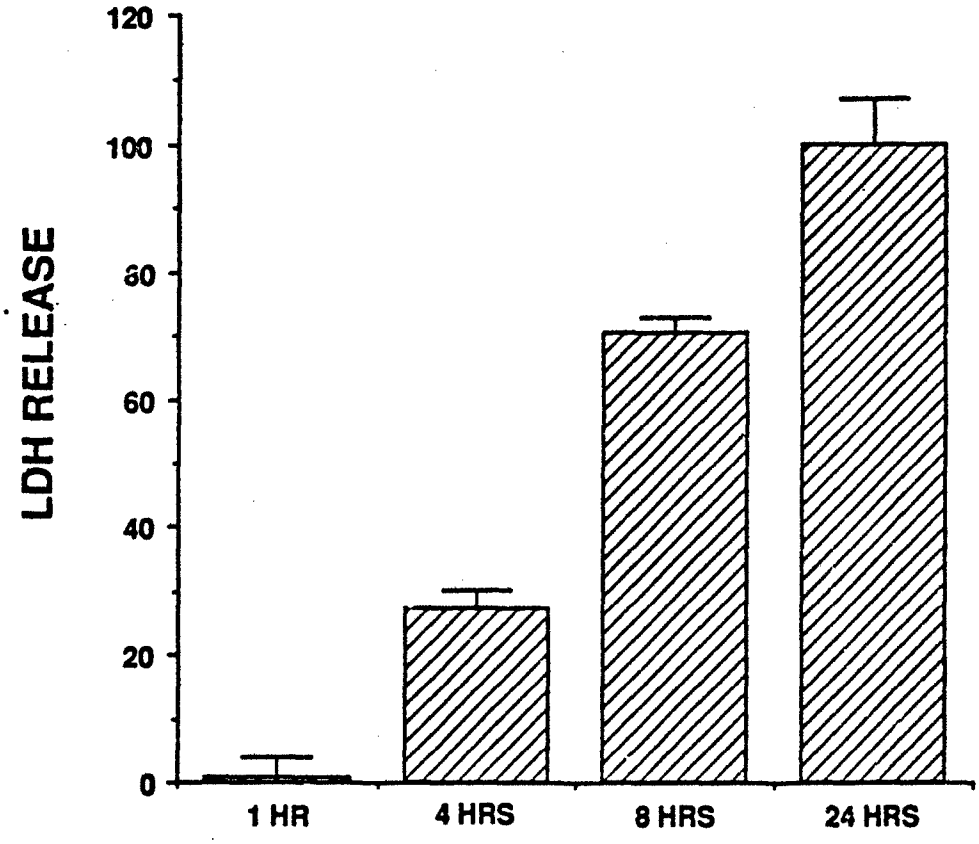
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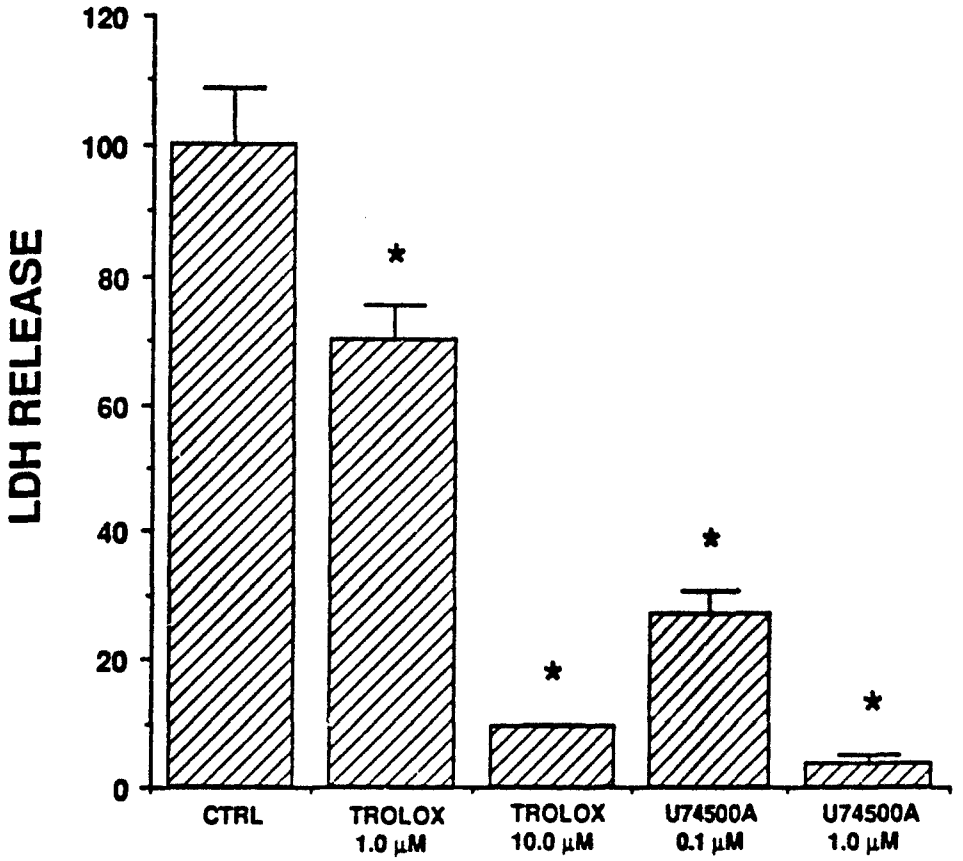
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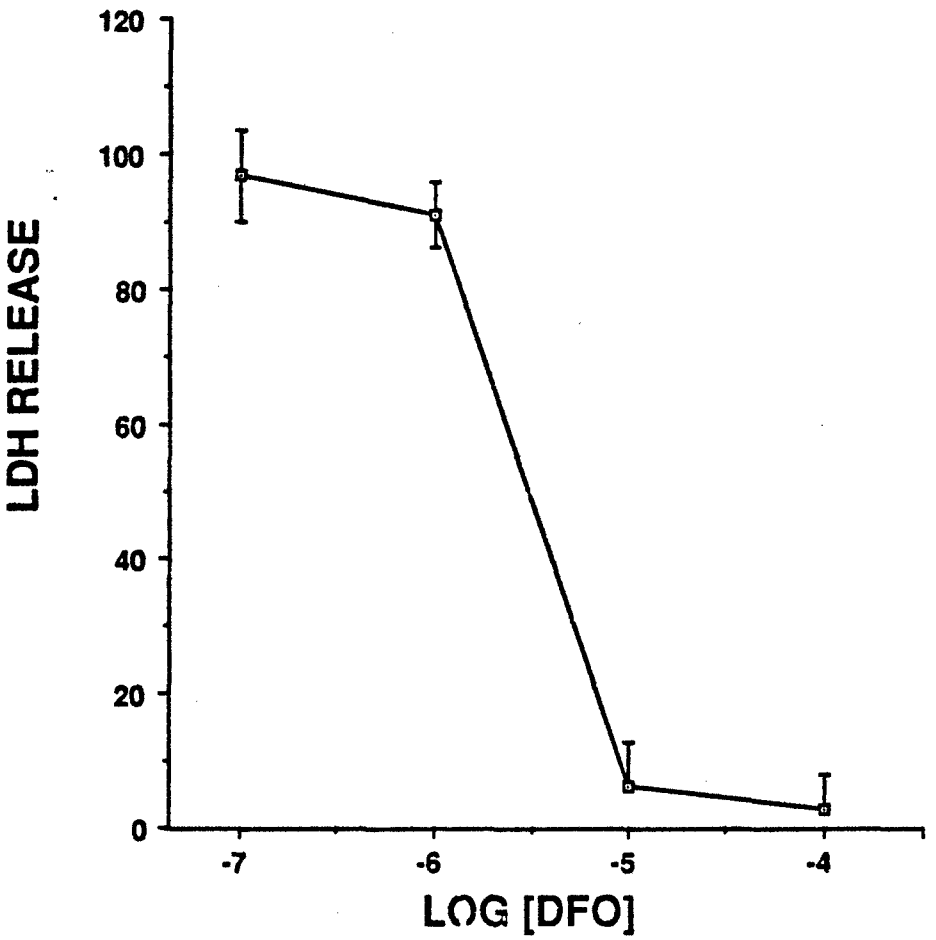
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HEMOGLOBIN CAUSES NEURONAL DEATH IN CORTICAL CELL CULTURES

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Key Words: hemoglobin, neuronal culture, iron, antioxidants, chelators, toxicity, deferoxamine, trolox

SUMMARY:

Hemoglobin (Hb) has been demonstrated to be neurotoxic when injected into the cerebral cortex, in vivo. However, associated systemic factors such as ischemia and epileptogenesis have limited investigations of Hb toxicity in the intact central nervous system (CNS). In this study, the neurotoxicity of human adult Hb was assessed in mixed neuronal and glial neocortical cell cultures derived from fetal mice. Exposure of cultures to hemoglobin for 24-28 hours produced widespread and concentration dependent neuronal death (EC_{50} 5-10 μM), without injuring glia. Brief exposures (1-2 hours) were well tolerated. Neuronal death was completely blocked by the 21-aminosteroid U74500A, the antioxidant Trolox, and the ferric iron chelator deferoxamine. The results of these experiments suggest that, in this system, hemoglobin is a potent neurotoxin and that hemoglobin neurotoxicity may contribute to secondary injury processes after trauma and intracranial hemorrhage.

Exposure of central neurons to extravascular blood is a feature common to many acute injury processes, including intraparenchymal hemorrhages, contusions, and hemorrhagic infarctions. Although much of the neuronal death occurring in these processes has been attributed to the primary injury or to secondary ischemia, a direct neurotoxic effect of blood has been hypothesized [19, 20]. A possible mediator of any such neurotoxicity is hemoglobin, which is present in erythrocytes in high concentration and accounts for most of the 8-10 millimolar iron concentration in whole blood.

Under normal circumstances, any free hemoglobin is effectively sequestered by haptoglobin, and toxicity is likely attenuated [8, 9] . However, the release of large amounts of hemoglobin from lysed erythrocytes may saturate haptoglobin in the cellular microenvironment, and neurons and glia may be directly exposed to hemoglobin and its breakdown products. In vivo studies support a possible neurotoxic effect of parenchymal hemoglobin; in rats, intracortical injection produces cavitary lesions and gliosis at injection sites [19]. However, hemoglobin neurotoxicity is difficult to quantify in such in vivo systems due to the potent vasospasm [16] and seizure activity [19] that may accompany its administration. In the present study, we utilized a primary murine neocortical cell culture system, in order to investigate hemoglobin neurotoxicity in a highly controlled environment free of complicating systemic variables.

All hemoglobin solutions were prepared in the pilot-plant production facility at the Letterman Army Institute of Research. Human Ao hemoglobin was purified by high pressure liquid chromatography according to the technique of Christensen et al. [5]. Hemoglobin concentrations were determined using Drabkin's solution [24] and methemoglobin concentrations were determined according to the method of Evelyn and Malloy [6, 11] to be less than 4.0%. All hemoglobin concentrations are expressed as the concentration of heme, or hemoglobin monomer.

Mixed cortical cell cultures, containing both neurons and glia, were prepared with modification of methods described by Choi et al. [4]. After halothane anesthesia, pregnant Swiss-Webster mice (15-16 days gestation) were euthanized by cervical dislocation. Embryos were rapidly removed and decapitated, and the neocortex was dissected free and incubated in media containing 0.09% acetylated trypsin at 37°C for one hour. Tissue was then suspended in plating media consisting of Eagle's minimal essential medium, 5% heat-inactivated horse serum, 5% fetal bovine serum, glutamine (2mM), and glucose (21

mM). After trituration through a flame-polished Pasteur pipette, cells were diluted in additional plating media and plated on confluent glial cultures in 15 mm multiwell plates (2.2 hemispheres/plate). Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Culture media was partially changed twice weekly with media identical to plating media but lacking fetal serum. Nonneuronal cell division was inhibited at 6-9 days in vitro (DIV) by addition of 10^{-5} M cytosine arabinoside. Cortical glial cultures were prepared from neonatal Swiss-Webster mice. Dissection and dissociation were as described above for mixed cultures, except that plating media contained 10% fetal bovine serum, 10% horse serum, and epidermal growth factor (10 ng/ml). The cell suspension was plated on 15 mm Primaria (Falcon) multiwell plates at a density of 0.5 hemispheres per plate.

Exposure to hemoglobin was carried out in a defined solution consisting of MEM plus glucose (25 mM), which was substituted for culture media by triple exchange. After addition of hemoglobin alone or with drugs, cultures were incubated at 37°C in a 5% CO_2 atmosphere. Neuronal injury was estimated in all experiments by examination of cultures with phase-contrast microscopy at 100-200x, and was quantified by measurement of lactate dehydrogenase (LDH), released by injured and necrotic cells, in the culture media at the end of the exposure period [13]. Cultures were used for these experiments at 13-16 DIV.

Exposure of cultures to hemoglobin for 24-28 hours produced significant neuronal injury which was concentration dependent between 0.1 uM and 100 μM (EC_{50} 5-10 μM). Exposure to 100 μM hemoglobin resulted in degeneration of 80-100 % of neurons (Fig. 1). The underlying glial monolayer, in contrast, remained intact and appeared morphologically normal through several days of exposure. Experiments using pure glial cultures revealed no evidence of hemoglobin-induced glial injury or LDH release.

Brief exposure to hemoglobin was consistently well tolerated by both neurons and glia. A one hour incubation in the presence of 100 μM hemoglobin produced no significant neuronal injury over the subsequent 23 hours (Fig. 2). Some variation was noted in the exposure time required to produce neuronal injury, particularly between cultures prepared with different lots of serum. In most experiments, many neurons were swollen after an eight hour exposure period, and subsequently degenerated to debris over the next several hours.

In order to test the hypothesis that hemoglobin neurotoxicity is mediated by oxidation of cellular components, we exposed cultures to hemoglobin in the presence of Trolox or the

21-aminosteroid U74500A (Fig. 2). Trolox, a water-soluble analog of α-tocopherol [26], consistently attenuated the neuronal injury produced by 24-28 hour exposure to 100 μM hemoglobin, with near complete protection noted at 10 μM Trolox (Fig. 3). U74500A, an inhibitor of lipid peroxidation which may chelate iron [3], was also effective and more potent. Significant neuroprotection was noted at a concentration of 100 nM which was complete at 1 μM. At 10 nM, no effect was seen. U74500A was prepared in a stock solution containing dimethylsulfoxide (DMSO). No significant effect was noted from the DMSO vehicle alone at the concentration used in these experiments (0.1%).

Lipid peroxidation is likely initiated by highly reactive hydroxyl radical (OH·). Formation of OH· likely occurs via the iron-catalyzed Haber-Weiss reaction [2, Minotti, 1987 #46]. In order to assess the role of OH· formation in hemoglobin neurotoxicity, we used deferoxamine, which is a ferric iron chelator that also scavenges OH· [10]. Neuronal injury was blocked by deferroxamine in a concentration-dependent fashion, and complete protection was provided by 10 μM (Fig. 4).

The precise molecular events precipitating neuronal death after hemoglobin exposure remain undefined. Hemoglobin is capable of releasing reactive iron that can catalyze the formation of OH· [21, 7]. Once formed, OH· would most likely react in the immediate vicinity of the site of production and, if formed in the extracellular space, would have limited access to the intracellular organelles [23]. Interaction of OH· with cell membrane phospholipids may initiate free radical chain reactions within the membrane, leading to decreased membrane fluidity and loss of membrane integrity [22]. Alternatively, OH· may interfere with the activity of membrane-bound enzymes, either by direct protein oxidation or disruption of surrounding membrane phospholipids. Anderson and Means reported inhibition of spinal cord Na^+/K^+ ATPase in vivo by iron salts, which was prevented by high dose methylprednisolone, α-tocopherol, or selenium [1]. Leclerc et al., noted potent inhibition of erythrocyte membrane Ca^{2+}/Mg^{2+}ATPase in vitro by 100 μM ferric heme or nonheme iron [14, 15]. Even partial inhibition of membrane cation pumps would likely have dire consequences for neurons, which must maintain steep ionic gradients and recover promptly from depolarization.

Regardless of the exact mechanism of hemoglobin-dependent CNS damage, the results of the current experiments suggest that exposure of the neurons to hemoglobin may

be undesirable and that it may beneficial to clear hemoglobin from extravascular spaces as rapidly as possible. A normal mechanism of hemoglobin clearence is mediated by the plasma protein haptoglobin, which forms an irreversible complex with hemoglobin and facilitates its uptake by the liver [12]. This clearence process is extremely efficient: the plasma half-life of haptoglobin alone in human plasma is 3.5 days [12]. However, when bound to hemoglobin, the plasma half-life of the complex is approximately 10 minutes [12]. In addition to assisting the clearence of free hemoglobin, haptoglobin also minimizes or completely blocks the pro-oxidant effects of hemoglobin [8]. Thus, haptoglobin may play an important role in the control of hemoglobin-dependent tissue damage.

In the present experiments, exposure of cultures to a hemoglobin concentration approximating 1% of that present in human blood produced marked neuronal injury. These results should be interpolated to situations in vivo with some caution. During the course of these experiments, neurons were deprived of the usual antioxidant defenses normally present in extracellular fluid, e.g. plasma [25]. The presence of these constituents may counterbalance the loss of endogenous CNS antioxidants such as reduced glutathione, α-tocopherol, and ascorbate, all of which decline precipitously after trauma and ischemia [17, 18, 27]. However, high hemoglobin concentrations may eventually overwhelm any remaining endogenous defenses and produce neurotoxicity. Pharmacologic strategies aimed at attenuating such injury may be of value in the management of CNS trauma and hemorrhage.

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FIGURE LEGENDS

Fig. 1. Concentration dependence of hemoglobin neurotoxicity. Sister cultures were exposed to indicated concentrations of hemoglobin for 24-28 hours. LDH in the culture media was measured at the end of the experiment (mean + S.E.M., n = 8 cultures for each condition). LDH in this and subsequent figures was scaled to the mean value released by cultures exposed to hemoglobin 100 μM (=100). Asterisk indicates significant difference from sham wash control ($P < 0.05$, Student-Newman-Keuls test).

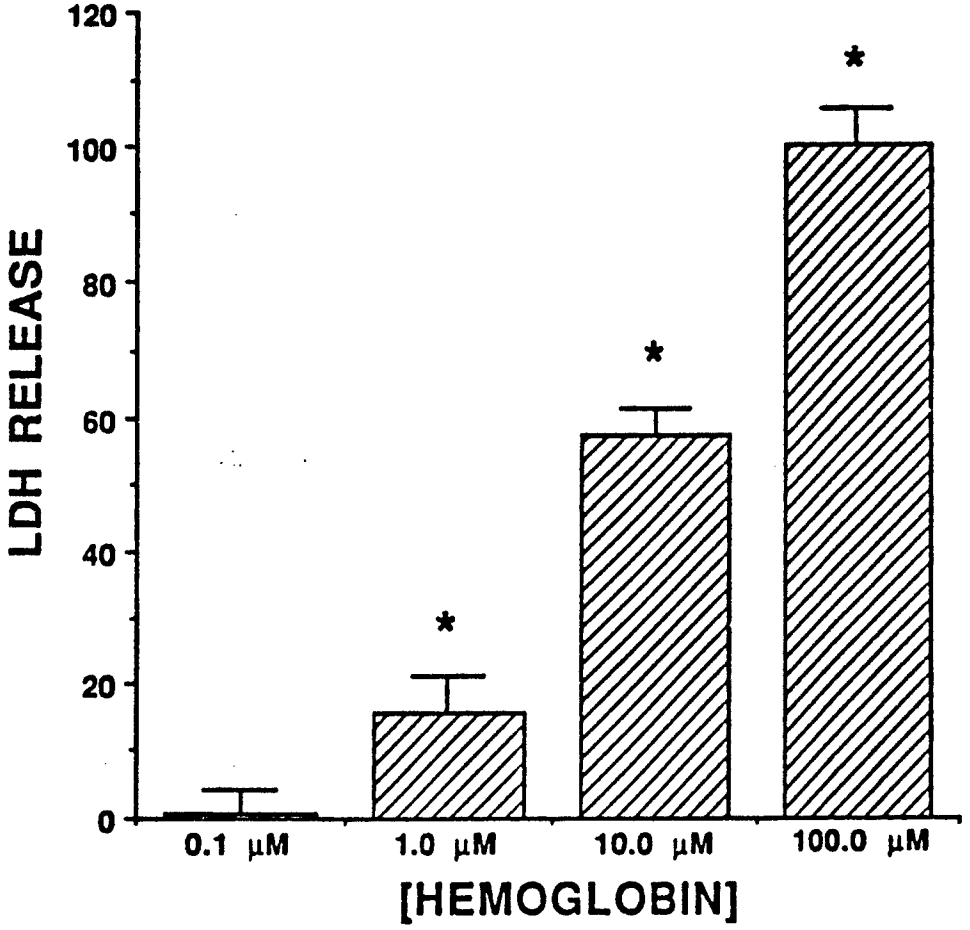
Fig. 2. Time course of hemoglobin neurotoxicity. Sister cultures were exposed to hemoglobin 100 μM for indicated time. LDH in the culture media was measured prior to washout and at 24 hours, and results were summed (mean + S.E.M., n = 4 for each condition).

Fig. 3. Antioxidant blockade of hemoglobin neurotoxicity. Sister cultures were exposed to hemoglobin 100 μM for 24-28 hours either alone (CTRL) or in the presence of indicated concentrations of trolox or U74500A. LDH in the culture media was measured at the end of the exposure period (mean + S.E.M., n = 8 for each condition). Asterisk indicates significant difference from control ($p < 0.05$, Student-Newman-Keuls test).

Fig. 4. Iron dependence of hemoglobin neurotoxicity. Media LDH (mean +/- S.E.M., n = 8) in sister cultures after 24-28 hour exposure to hemoglobin 100 μM in the presence of indicated concentrations of deferoxamine, scaled to LDH released in control cultures exposed to hemoglobin alone (=100). Approximate IC_{50} for deferoxamine = 3μM.

The opinions and assertions contained herein are the private views of the authors and are not to be construed as official, nor do they reflect the views of the Dapartment of the Army or the Department of Defense (AR 360-5).

The experimental studies of the authors described in this report were reviewed and approved by the Institutional Review Committee/Animal Care and Use Committee at Letterman Army Institute of Research. The manuscript was peer reviewed for compliance prior to submission for publication. In conducting the research described here, the author adhered to the "Guide for the Care and Use of Laboratory Animals," DHEW Publication (NIH) 85-23.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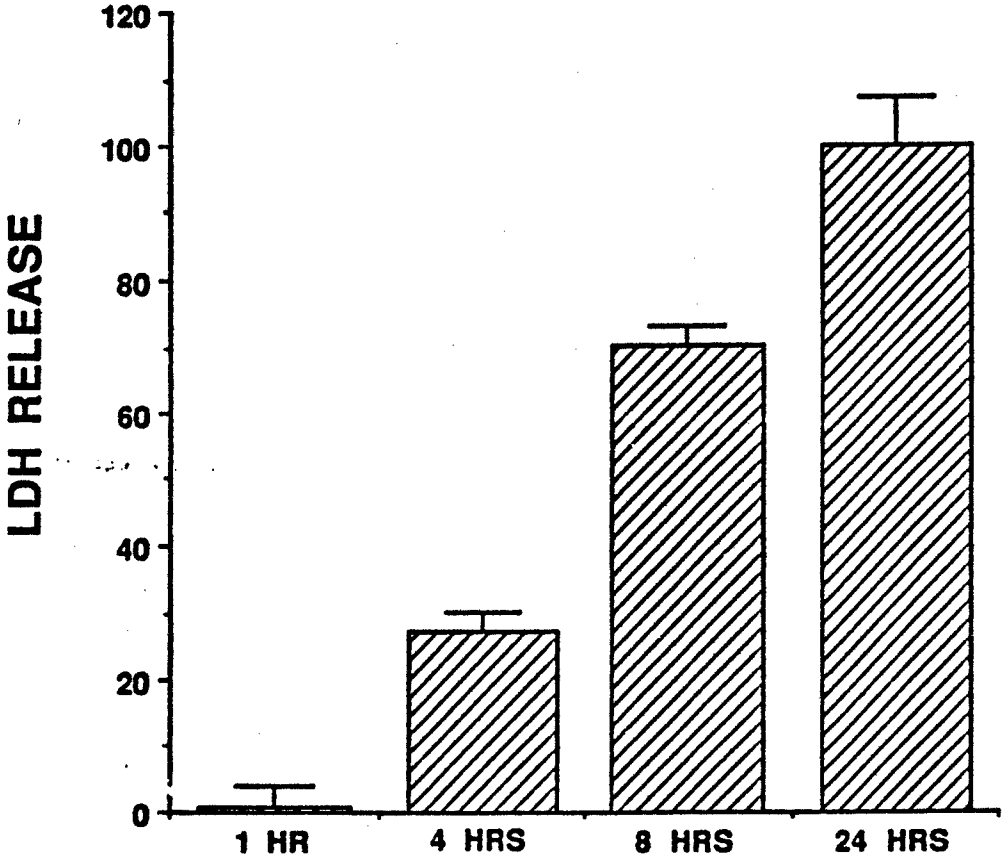
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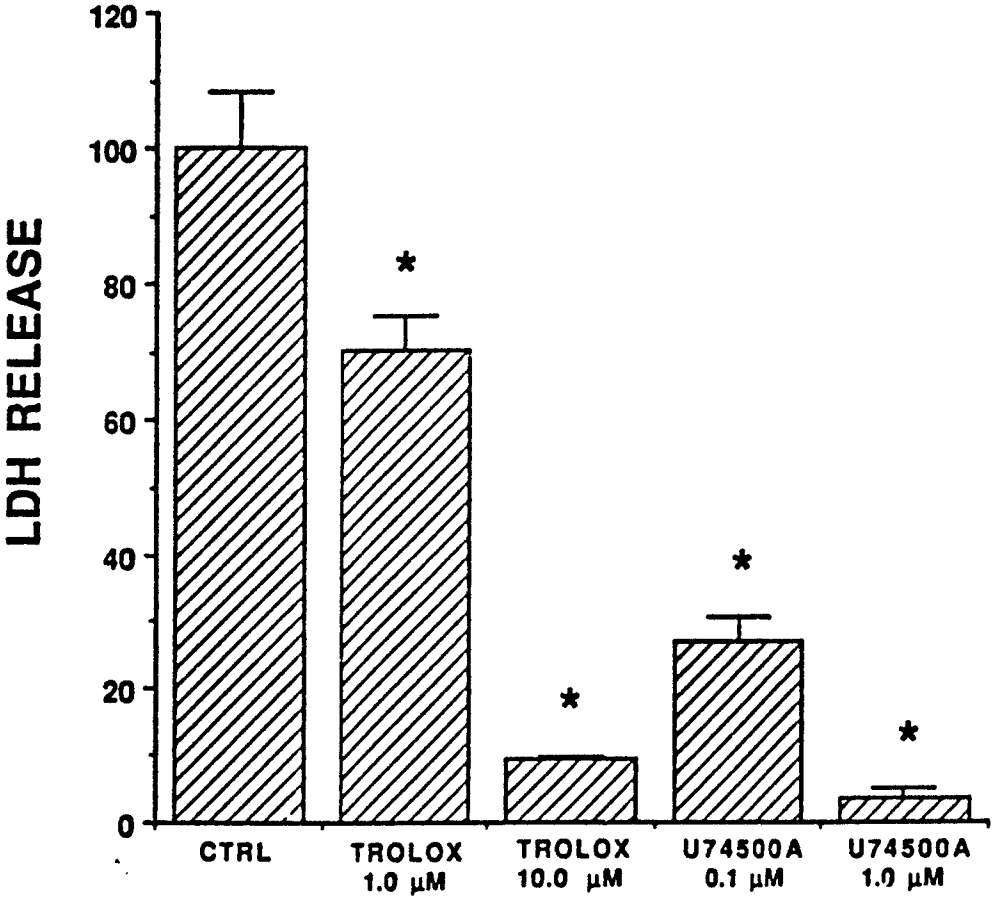
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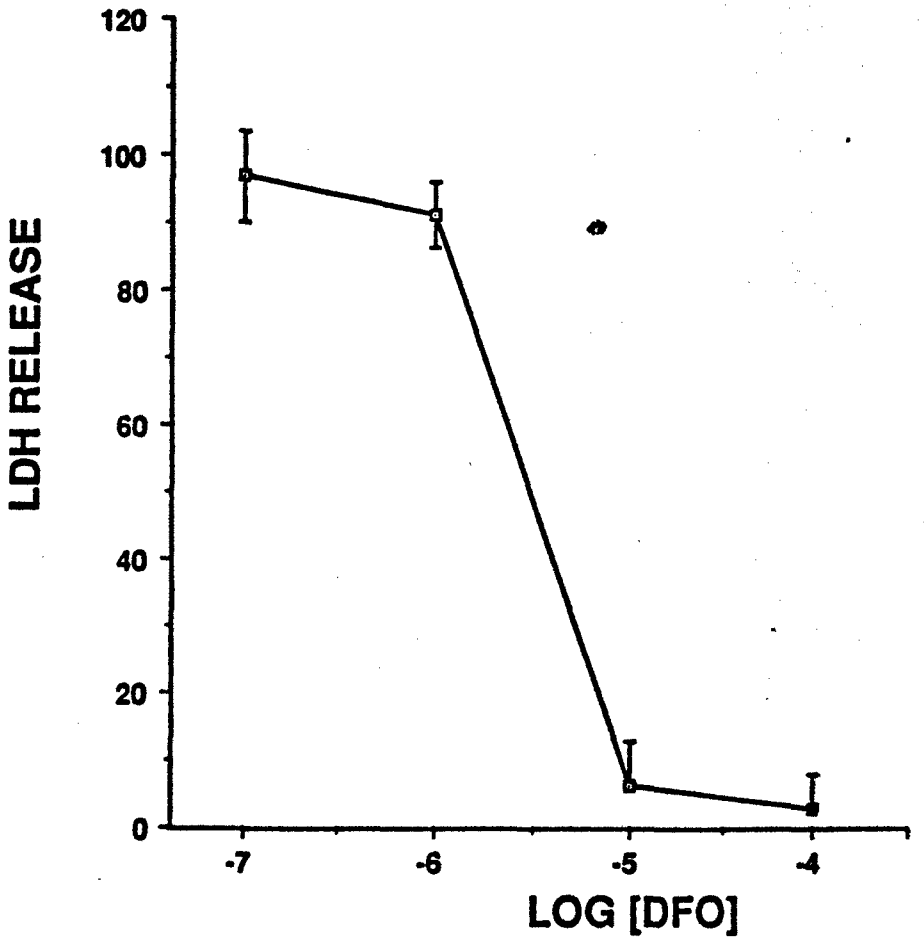
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